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Determination of testosterone concentrations in rat plasma using liquid chromatography–atmospheric pressure chemical ionization mass spectrometry combined with ethyl oxime and acetyl ester derivatization

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Abstract

A quantitative method for measuring testosterone (T) concentrations in rat plasma was developed using ethyl oxime and acetyl ester derivatization and liquid chromatography–atmosphere pressure chemical ionization tandem mass spectrometry (LC–APCI-MS/MS). The method utilizes a solid phase extraction with Varian Bond Elut C18, a derivatization process to form testosterone ethoxime acetate and LC–APCI-MS/MS with a reversed phase LC and a C8 column. This method is capable of detecting testosterone concentrations as low as 0.2 ng/ml in a 0.05 ml sample of rat plasma. This method can be used as a sensitive chromatography-based assay for small sample volumes of rat blood.
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Keywords: Testosterone; Liquid chromatography–atmosphere pressure chemical ionization mass spectrometry; Derivatization; Acetate; Oxime

1. Introduction

Testosterone (T) is one of most important and most commonly measured sex hormones. T measurements are often performed using radioimmunoassays (RIAs). RIAs are simple, rapid, and inexpensive, but crossreactivity in these assays is of great concern [1]. In contrast, chromatography-coupled mass spectrometric methods, especially LC-MS/MS methods, have high specificity and involve little or no crossreactivity [1–4].

In the field of pharmaceutical research, serum or plasma T concentrations are quantified as therapeutic markers for hormone dependent diseases such as prostate cancer [5]. For research purposes, small experimental animals such as rats are often used [6]. As a result, smaller plasma volumes are available for analysis. Many of the reported LC-MS methods have been developed for the analysis of human serum or plasma [1,2], and subsequently they utilize serum or plasma sample volumes around 1 ml or larger. The only exception to these LC-MS methods that use relatively large sample volumes is that reported by Zhao et al. [3]. They utilized only 20 μl of human testicular fluid and their LC-electrospray ionization (ESI)-MS/MS technique seems very promising in terms of its sensitivity. However, detailed validation data with human plasma has yet to be presented.

To reduce plasma volume for analysis, an increase in assay sensitivity is essential. Atmospheric pressure chemical ionization (APCI)-MS/MS is an attractive technique because of its low matrix effects [7]. Indeed, it can be argued that ESI technique is advantageous for its excellent sensitivity for T with current turboionspray ion sources, judging from some reports [1,3,4], but the sensitivity issue of APCI analysis for T can be improved to adequate level by chemical derivatization. T is a 3-keto, 17-hydroxy steroid and its ionization efficiency is not very high under APCI conditions because it has no chemical groups with high proton affinity. Researchers have reported that oxime derivatization [8,9] or acetyl ester

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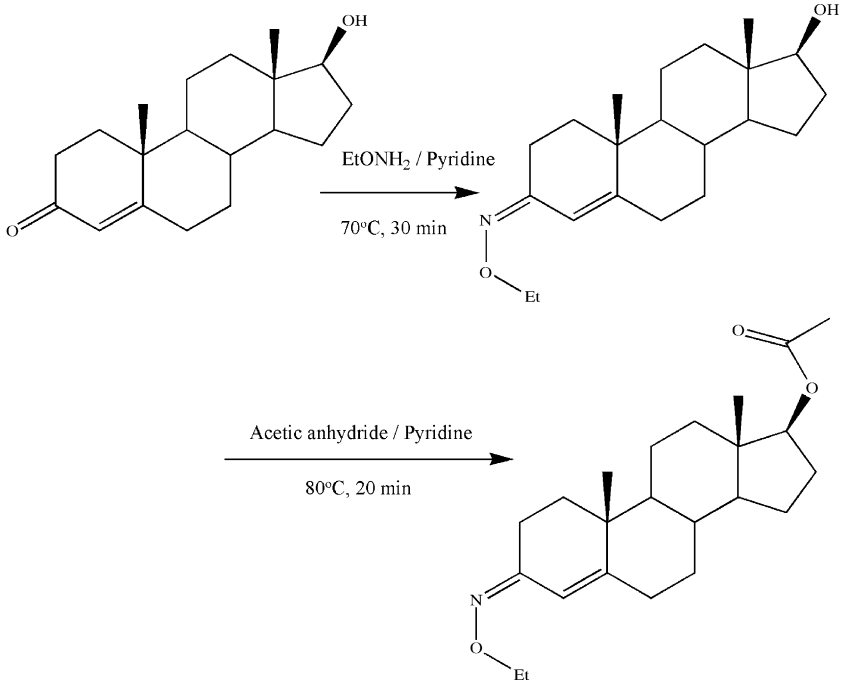

Fig. 1. The reaction scheme of ethyloxime acetate derivatization of T.

derivatization [8,10] can increase proton affinity and subsequently APCI sensitivity. In this study, an LC–APCI-MS/MS method for an ethoxime acetate derivative (Fig. 1) of testosterone, utilizing only 0.05 ml of plasma, was developed and validated.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade methanol, analytical grade acetic anhydride, and guaranteed reagent grade ammonium acetate were purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan). Sequential grade pyridine was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Ethoxyamine hydrochloride was purchased from Acros Organics BVBA (Belgium). Purified water was prepared using a Mill-Q water purification system (Millipore, Billerica, MA, USA).

2.2. Synthesis of an internal standard

[$^2H_4$, ^{18}O]-T, which was used as an internal standard (I.S.), was synthesized in our laboratory in a previous testosterone metabolic study [11] for use as a stable isotope-labeled tracer. Briefly, [$^2H_3$]-4-androstene-3,17-dione with a ^2H-labeled 19-methyl group, was synthesized from 5(10)estrene-3,17-dione, according to the method described by Baba et al. [12]. Subsequently, [$^2H_3$]-4-androstene-3,17-dione was treated with $Li^{18}OH$, and the oxygen atoms on C-3 and C-17 were replaced with ^{18}O. Next, the C-3 and C-17 carbonyl groups were reduced with $NaBD_4$, and then the C-3 hydroxyl group was selectively oxidized with dichlorodicyanobenzoquinone. The final product was [$^2H_4$, ^{18}O]-T with $^2H_3$ on the 19-methyl group, and ^2H and ^{18}O on the C-17 position.

2.3. Samples

Female rat plasma, used as a blank plasma matrix, was purchased from Nihon Charles Liver Inc. (Yokohama, Japan). Plasma was prepared at Nihon Charles Liver Inc., by collecting blood from the abdominal arteries of 9–12-week-old female Crl:CD(SD) rats under ethyl ether anesthesia, using heparin as an anticoagulant. Plasma was prepared by subsequent centrifugation. In the application part of our study, blood samples (200 μl) were collected from the jugular veins of 8-week-old male Crl:CD(SD) rats, using heparin as an anticoagulant. These blood samples were then centrifuged (1800 g, 10 min, 4 °C) to extract blood plasma. Rats were housed for 12 days prior to the experiment, under controlled temperature and lighting (lights on 07:00–19:00 h).

2.4. Sample preparation

To each 50 μl plasma sample, 20 μl of I.S. methanol solution (containing 1 ng of I.S.) was added. The mixture was then diluted with 0.2 ml of water and applied to a Bond Elut C18 cartridge (200 mg, Varian Instruments, Walnut Creek, CA, USA) that was preconditioned with 3 ml of methanol and 3 ml of water. The cartridge was washed with 1 ml of

water and 1 ml of methanol:water (1:1, v/v), and eluted with 1 ml of methanol. The mixture was evaporated to dryness under reduced pressure at 40 °C. To each of the extracted samples, 50 μl of 20 mg/ml ethoxyamine hydrochloride in pyridine was added. The mixture was left to react at 70 °C for 30 min. Ethanol (0.5 ml) was added to the sample to make an azeotropic mixture with pyridine, and the solvent was evaporated to dryness under reduced pressure at 40 °C. Acetic anhydride (20 μl) and pyridine (20 μl) were added to the sample and the reaction was performed at 80 °C for 20 min. Ethanol (0.5 ml) was added to the sample and the solvent was evaporated to dryness under reduced pressure at 40 °C. The derivatized samples were dissolved in 100 μl of methanol and filtered through an Ultrafree-MC LG hydrophilic PTFE filter (0.22 μm, Millipore, Billerica, MA, USA). A 30 μl portion of the filtrate was subsequently subjected to LC-MS/MS analysis.

2.5. Extraction efficiency

Extraction efficiency was measured using the internal standard method.

For T, three concentrations of extracted samples ($n = 3$ per concentration) were prepared by extracting the female rat plasma spiked with T (0.2, 2, or 16 ng/ml). After extraction, I.S. (20 ng/ml equivalent) was added to samples as internal standard for extraction. Reference samples were prepared by mixing blank plasma extracts with T (0.2, 2, or 16 ng/ml) and I.S. (20 ng/ml). Both series of samples were further derivatized by the procedures described in Section 2.4. The extraction efficiency of T was calculated by taking the peak area ratio (T/I.S.) of each extracted sample and finding its percentage of the peak area ratio of our reference samples.

For I.S., one concentration of extracted samples ($n = 3$) were prepared by extracting the female rat plasma spiked with I.S. (20 ng/ml). After extraction, T (2 ng/ml equivalent) was added to I.S. samples (as internal standard for extraction). Reference samples were prepared by mixing blank plasma extracts with T (2 ng/ml) and I.S. (20 ng/ml). Both series of samples were further derivatized using the procedures described in Section 2.4. I.S. extraction efficiency was calculated by taking the peak area ratio (I.S./T) of each extracted sample and finding its percentage of the peak area ratio of our reference samples.

2.6. Matrix effects

Matrix effects were investigated by comparing the response of standard spiked plasma extracts (matrix samples) and the response of a neat standard solution.

For T, two concentrations of standard spiked plasma extracts ($n = 3$ per concentration) were prepared by spiking female rat plasma extracts with T (2 or 16 ng/ml). Reference neat samples were prepared by diluting T to desired concentration (2 or 16 ng/ml equivalent). Both series of samples were further derivatized using the procedures described in Section 2.4. Matrix effects were evaluated by taking the peak area of each standard spiked plasma extract (female endogenous T concentration subtracted according to duplicate blank plasma samples) and finding its percentage of the peak area of reference neat samples.

For I.S., one concentration of standard spiked plasma extracts ($n = 3$ per concentration) were prepared by spiking female rat plasma extracts with I.S. (20 ng/ml). Reference neat samples were prepared by diluting I.S. to desired concentration (20 ng/ml equivalent). Both series of samples were further derivatized using the procedures described in Section 2.4. Matrix effects were evaluated by taking the peak area of each standard spiked plasma extract and finding its percentage of the peak area of reference neat samples.

2.7. Calibration curve and quantification

Calibration standards were prepared by adding 20 μl of T methanol solution containing 0, 0.01, 0.02, 0.04, 0.1, 0.2, 0.4, and 1 ng of T to 50 μl of water. As a result, the calibration points were 0, 0.2, 0.4, 0.8, 2, 4, 8, and 20 ng/ml. At first, to investigate matrix (plasma) effects on quantification, calibration curves with female rat blank plasma were also constructed. In this case, calibration standards were prepared by adding T methanol solution to 50 μl of female rat blank plasma.

The calibration curve $y = mx + b$ was obtained by assigning the concentration of T and the peak area ratio of T to I.S. to x and y, respectively. Subsequently, a $1/x$ weighted linear regression was performed.

2.8. LC-MS/MS

The LC-MS/MS system consisted of an LC10A-VP HPLC system with a SIL-HT automatic sample injector (Shimadzu, Kyoto, Japan) and an API 4000 LC-MS/MS system (Applied Biosystems, Lincoln Centre Drive, Foster City, CA, USA). The LC-MS/MS system was controlled with Analyst 1.3 software (Applied Biosystems). The mobile phase was 10 mM aqueous ammonium acetate: methanol (10:90, v/v) and delivered at the flow rate of 1 ml/min. The analytical column was a Unison UK-C8 (75 mm length × 4.6 mm I.D., particle size 3 μm, pore size 13 nm, Imtakt Corporation, Kyoto, Japan). No guard column was used in this study. The mass spectrometer was operated in APCI mode with positive ion detection. The mass spectrometric parameters were as follows: a Corona current of 3 μA; a declustering potential of 100 V; a collision energy of 40 eV; a collision cell exit potential (CXP) of 10 V; and an ion source temperature of 450 °C. The collision and nebulizer gases were nitrogen and air, respectively, and their parameters were 6 and 20. For quantitative analysis, multiple reaction monitoring (MRM) transitions of 374.5 → 152.4 (T) and 380.5 → 152.1 (I.S.) were monitored with dwell times of 500 ms.

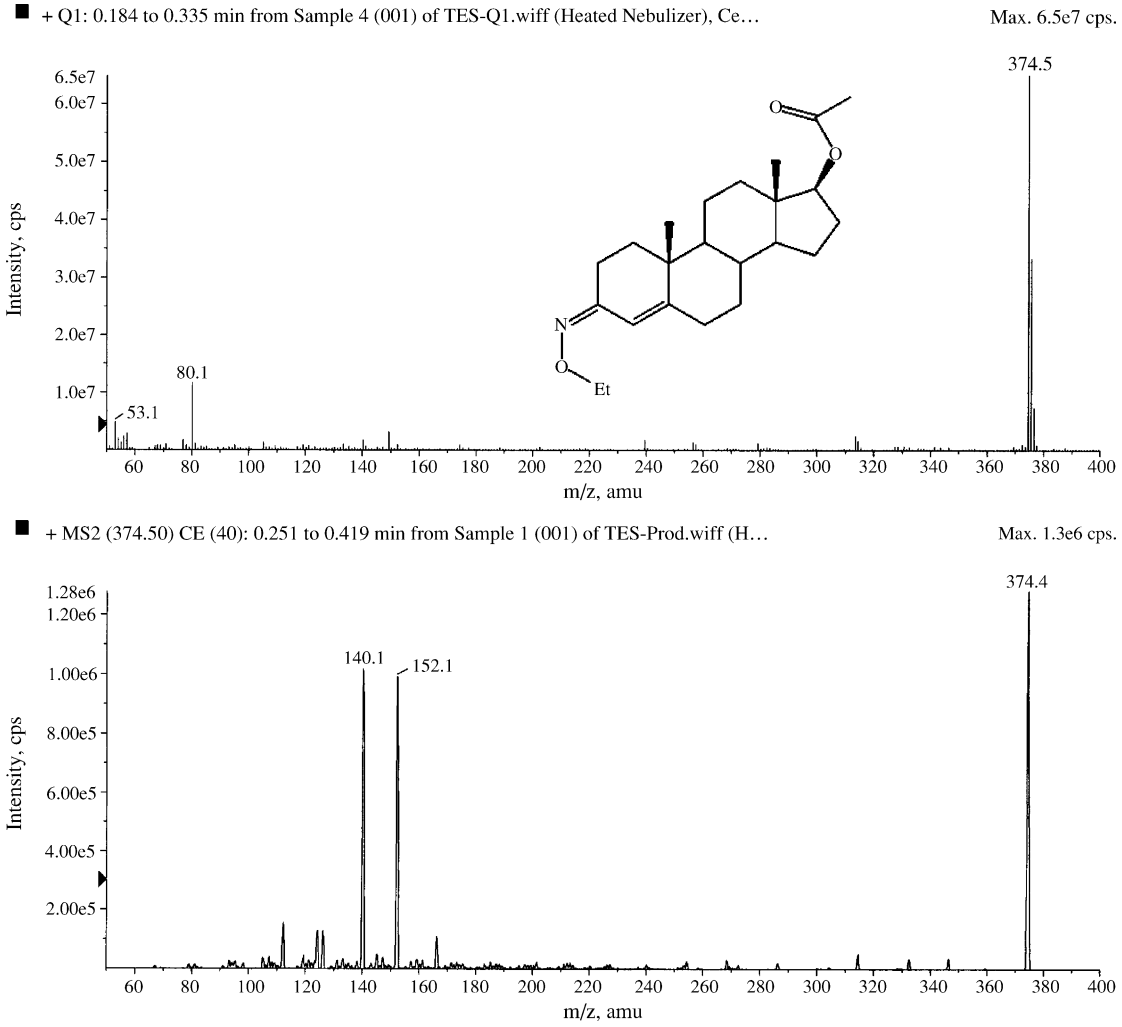

Fig. 2. APCI-MS spectrum (upper) and product ion spectrum (lower) of testosterone ethoxime acetate derivative.

3. Results and discussion

3.1. Mass spectrum

Fig. 2 shows the mass spectrum of T ethoxime acetate with APCI ionization. A molecular-related ion of *m/z* 374.5, $[M + H]^+$ of T ethoxime acetate was observed. In the product ion scan mode, *m/z* 140.2 and 152.2 were observed. The mass spectrum of I.S. is also shown in Fig. 3. Major transitions of 374.5 → 152.4 (T) and 380.5 → 152.1 (I.S.) were selected to monitor MRM.

3.2. Comparison of ethoxime and methoxime derivatives

Acetate alkyl oxime derivatization was selected because it was reported to increase the APCI sensitivity of dehydroepiandrosterone [10], which is physico-chemically similar to T.

In the method development phase, two commercially available oxime reagents, methoxyamine hydrochloride and ethoxyamine hydrochloride, were tested. Because the ethoxime acetate derivative yielded more peak counts (with 300 pg of T, methoxime acetate gave 334 ± 78 peak counts, whereas ethoxime acetate gave 1450 ± 190 peak counts (average ± S.D., $n = 3$)), our quantification method was developed using the ethoxime acetate derivative.

3.3. Extraction procedure and extraction efficiency

For the extraction procedure, solid phase extraction using a silica-based C18 cartridge was chosen because it is known for its usefulness in extracting steroids [9,13–15].

In the method development phase, recovery experiments using [^{14}C]-testosterone (synthesized in our laboratory, sample size 0.1 μg/1 kBq/cartridge) were performed. The radioactivity was counted using a LSC-1500 liquid scintillation counter (Aloka, Tokyo, Japan) and Atomlight (Perkin-Elmer Inc., Wellesley, MA, USA) as liquid scintillation cocktail. The [^{14}C]-testosterone recovery percentages of the (1:9), (2:8), (3:7), (4:6), (5:5), (6:4), (7:3), (8:2), (9:1), and (10:0, v/v) water:methanol fractions were 0.2, 0.2, 0.2, 0.2, 0.3, 39.6, 56.0, 0.3, 0.2, and 0.1%, respectively. This result indi-

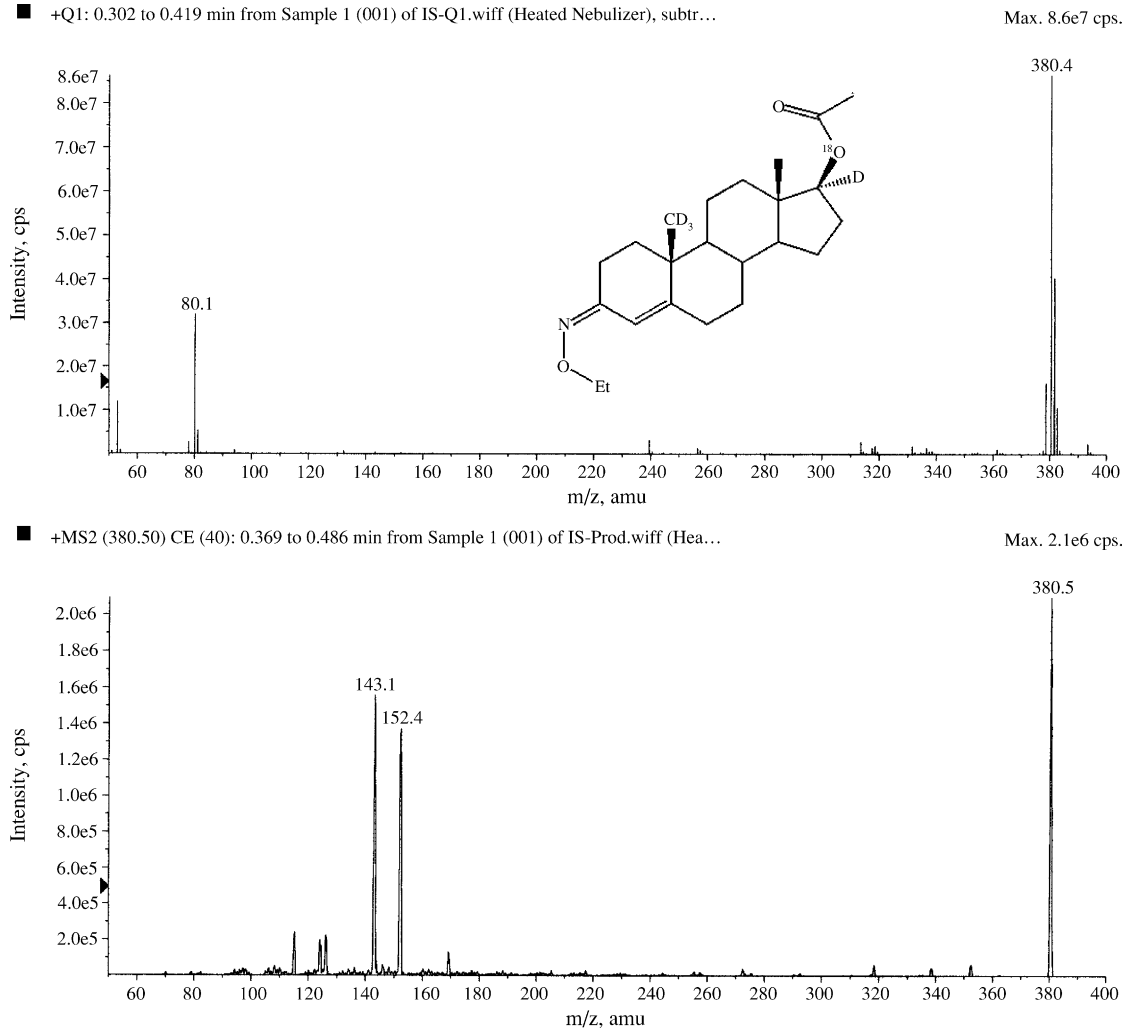

Fig. 3. APCI-MS spectrum (upper) and product ion spectrum (lower) of the internal standard, [$^{2}H_4$, ^{18}O]-testosterone ethoxime acetate derivative.

cated that [^{14}C]-testosterone was retained on the Bond Elut C18 column up to a water:methanol composition of 5:5 (v/v), and that testosterone could be completely recovered using methanol. As a result, the water:methanol system was chosen as a final extraction method.

As an alternative method aimed at more selective extraction, aqueous solution loading, *n*-hexane flushing [15] and subsequent *n*-hexane:ethyl acetate extraction was also performed. This technique resulted in leakage (6.3%) during hexane flushing and was not chosen as a final extraction method. The recovery of [^{14}C]-testosterone in the major fraction, *n*-hexane:ethyl acetate (7:3, v/v), was 87.9%.

In this validation study, the recovery of T in the solid phase extraction was found to be 105.7 ± 7.1, 104.5 ± 2.0, and 103.3 ± 4.4% (average ± S.D., $n = 3$), at concentrations of 0.2, 2, and 16 ng/ml, respectively. The recovery of I.S. was found to be 104.7 ± 6.9% (average ± S.D., $n = 3$) at 20 ng/ml. This data indicates complete recovery of T and I.S. during the solid phase extraction procedure.

3.4. Matrix effects

For T, the percentages of the matrix sample peak areas relative to the neat sample peak areas were 103.0% at 2 ng/ml and 108.8% at 16 ng/ml (average, $n = 3$). For I.S., the percentage of the matrix sample peak area relative to the neat sample peak area was 96.4% at 20 ng/ml (average, $n = 3$). From these results, we concluded that matrix effects were negligible.

3.5. Chromatography

The methanol–aqueous ammonium acetate system was chosen as the mobile phase because it provided a higher absolute sensitivity for derivatized T than the acetonitrile–aqueous ammonium acetate system. The concentration of ammonium acetate was set at 10 mmol/l after confirming constant sensitivity over the concentration range of 1, 2, 5, 10, and 20 mmol/l.

MRM of T and I.S. of male rat plasma extracts are shown in Fig. 4. With a recently developed C8 column, possessing a

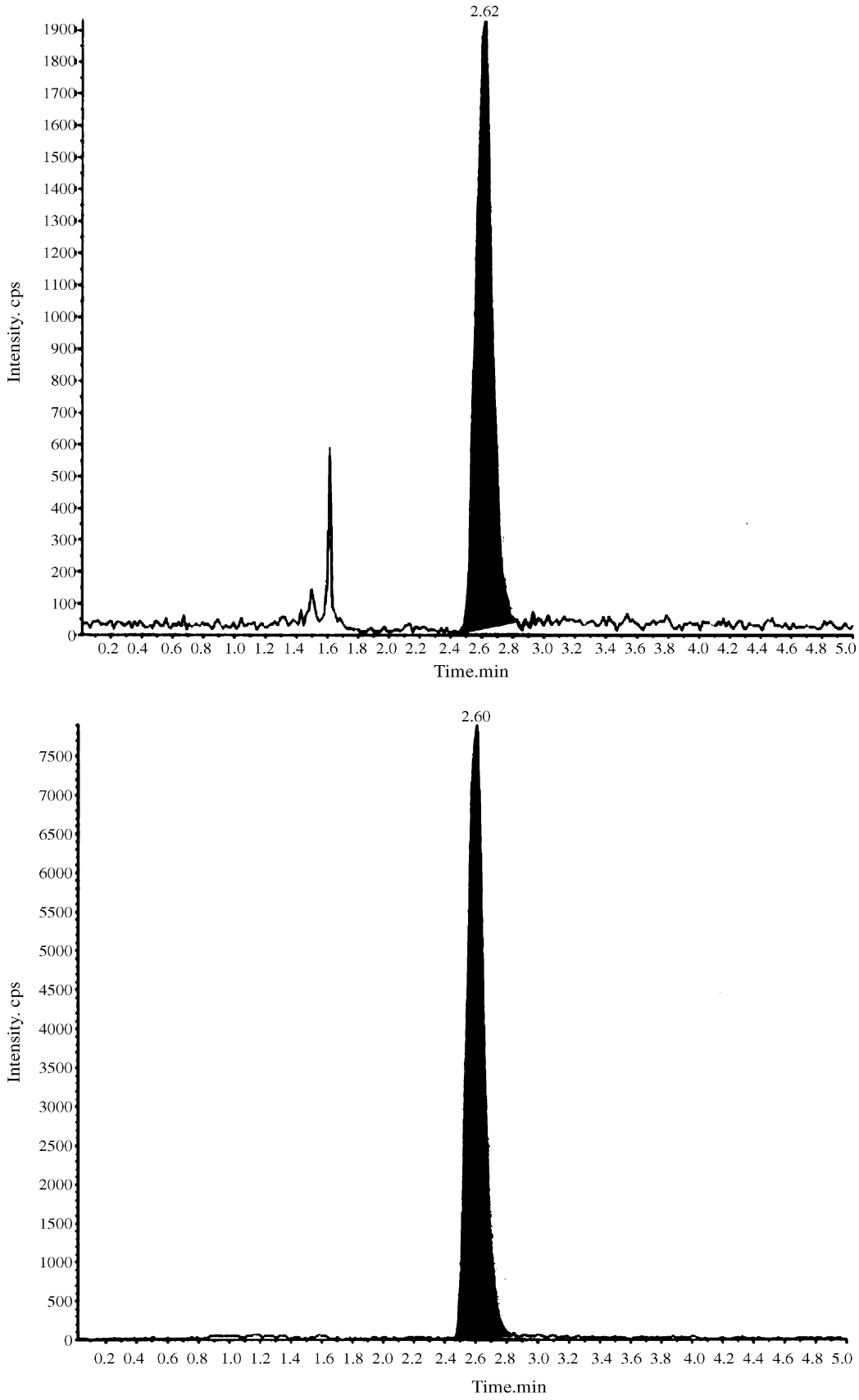

Fig. 4. Multiple reaction scans of *m*/*z* 374.5 → 152.4 (testosterone, upper) and *m*/*z* 380.5 → 152.1 ([$^{2}H_4$, ^{18}O]-testosterone, lower) of male rat plasma extracts. The plasma testosterone level of the rat was determined to be 5.87 ng/ml. The spiked I.S. concentration was 20 ng/ml.

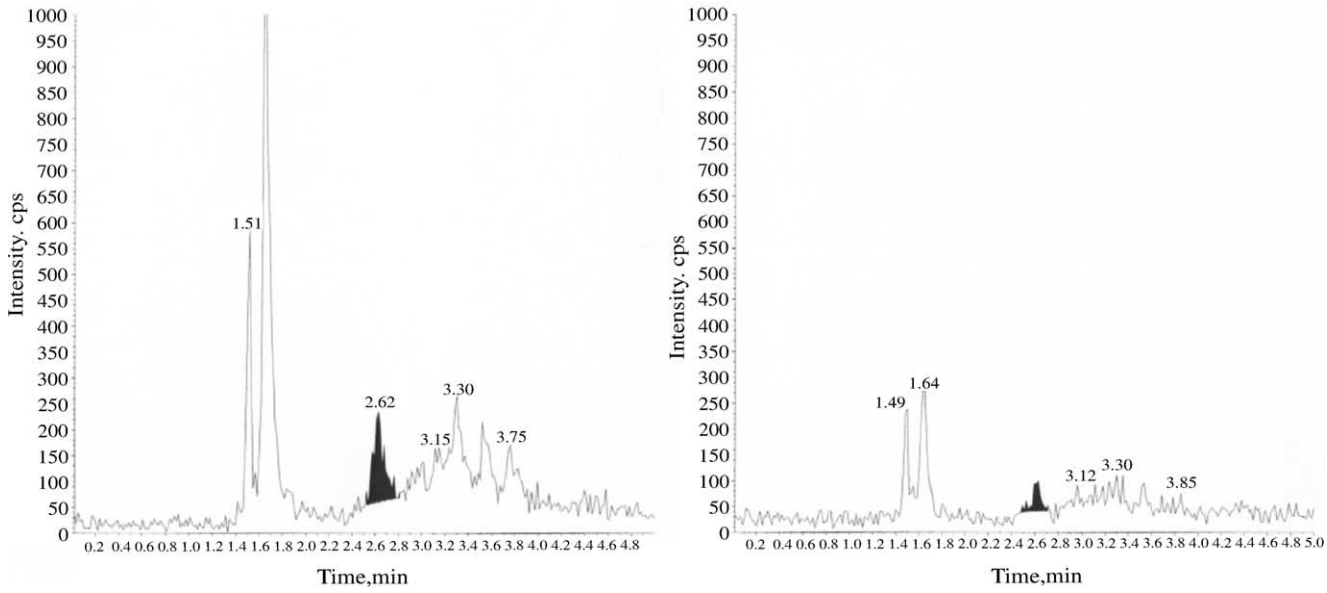

Fig. 5. Multiple reaction monitorings of *m/z* 374.5 → 152.4 (testosterone) of female rat plasma spiked with 0.2 ng/ml T (left) and female rat plasma extracts (right). Addition of 0.2 ng/ml T resulted in a clear response from the female internal T level in the monitoring.

polymeric end-capping feature and a 3 μm particle size, we obtained excellent peak shapes and our analysis was completed within 5 min. Although oxime derivatization results in the formation of anti- and syn-stereoisomers, they were not separated by the C8 column under our experimental conditions. The sensitivity of this method was not compromised because no split peaks were observed. Split peaks result in lower peak heights for each individual peak.

3.6. Calibration curves with and without plasma

To determine the extent to which plasma matrix affects quantification, calibration curves with and without plasma were compared in the same analytical run. The response function (*x*: added T concentration, *y*: peak area ratio) were $y = 0.0400x + 0.00356$ with plasma and $0.0405 + 0.00116$ without plasma. The slope of the curve was practically identical and only the intercept was different, reflecting the trace amount of T in female rat plasma. From these results, we concluded that plasma matrix did not affect the calibration curve and further study was performed using a calibration curve without plasma. In addition, linearity was confirmed in the range between the female blank concentration and 20 ng/ml.

3.7. Selectivity, accuracy, and precision

Fig. 5 shows the MRM scans of T-spiked (0.2 ng/ml) female rat plasma and "blank" female rat plasma. Adding 0.2 ng/ml of T resulted in a clearly distinguishable response from the "blank" female concentration in the chromatogram. The relationship between the blank concentration and 0.2 ng/ml spiked concentration was further investigated by comparing peak area ratios (T/I.S.). The peak area ratios were 5.3, 5.2, and 6.4 for three different batches (the average of duplicate analysis per batch) of female rat plasma, and assay selectivity was confirmed in a practical manner as the response at 0.2 ng/ml was five times that of the blank concentration.

Accuracy and precision was investigated by quantifying T-spiked female plasma samples using a non-matrix standard curve. A non-matrix standard curve was employed because standard curves in the presence and absence of a matrix were quite compatible in Section 3.4, and because it enabled direct quantification of T in plasma, without the use of techniques such as background-subtracted calibration or the use of charcoal stripped plasma. If a background-subtracted calibration curve is employed and there are meaningful batch-to-batch differences in the background, the LOQ of the assay may be affected because the lowest concentration of the calibration curve may change slightly. If charcoal stripped plasma is used in a validation study, proof of assay selectivity in the real plasma sample is difficult to obtain because some interference in plasma may be removed by charcoal. The problem with the present method is the estimation of a blank T concentration in female rat plasma during the validation study. Our blank T concentration was small, as described above, but it was high enough to influence the measurement accuracy of the lowest concentration sample (0.2 ng/ml). Because weighted regression was used in the construction of the calibration curve, estimation of the blank concentration (lower than the lowest calibration sample) by the calibration curve resulted in inaccurate results. To improve the situation, peak area ratio subtraction was used. The peak area ratio of a blank sample was subtracted from validation samples, and the blank-subtracted peak area ratio (*y*) was substituted into the linear equation $x = (y - b)/m$ (where x = concentration,

Table 1
Intra-day accuracy and precision of the T assay in T-spiked female rat plasma samples

T added (ng/ml)	Exogenous T found		
	Average (ng/ml)	R.S.D. (%)	Accuracy (%)
16.0	15.2	3.7	93.8
2.00	2.06	7.0	102.8
0.200	0.204	5.9	101.8

Data represents the results of $n = 5$ determinations of T-spiked female rat plasma within a single analytical run. Exogenous T was determined by substituting blank-subtracted peak area ratios of validation samples into the calibration equation. Accuracy was determined as a percentage of the ratio of determined exogenous T relative to the added T.

Table 2
Inter-day accuracy and precision of the T assay in T-spiked female rat plasma samples

T added (ng/ml)	Exogenous T found		
	Average (ng/ml)	R.S.D. (%)	Accuracy (%)
16.0	16.5	5.6	103.0
2.00	2.00	10.4	99.8
0.200	0.204	10.7	102.1

The data represents the results of duplicate determinations on each day for 4 days (total $n = 8$ determinations) of T-spiked female rat plasma. Exogenous T was determined by substituting blank-subtracted peak area ratios of validation samples into the calibration equation. Accuracy was determined as a percentage of the ratio of determined external T relative to the added T.

m = slope of the calibration curve, and b = intercept of the calibration curve).

The intra-day accuracy and precision are shown in Table 1. The intra-day accuracy percentage of exogenous T ranged from 93.8 to 102.8%, and R.S.D. ranged from 3.7 to 5.9%. The inter-day accuracy and precision are shown in Table 2. The inter-day accuracy percentage of exogenous T ranged from 99.8 to 103.0%, and R.S.D. ranged from 5.6 to 10.7%. From these results, intra- and inter-day reproducibility was found to be satisfactory.

From these results, the quantification limit for routine analysis was set at 0.2 ng/ml. This is the lowest calibration point at which selectivity, accuracy, and precision were confirmed with plasma-spiked samples.

3.8. Reproducibility of the calibration curve

The calibration curve parameters in the four analytical runs were as follows: the slope was 0.0341 ± 0.0045; the y-intercept was 0.00263 ± 0.00200; and the correlation coefficient was more than 0.9995.

3.9. Application

To investigate on the applicability of the present analysis method combined with repeated sampling, T concentrations in 8-weeks-old male rats ($n = 5$) were determined by repeated sampling from their jugular veins. The T concentrations were 4.97 ± 3.53 ng/ml at 12:00, 3.96 ± 1.97 ng/ml at 14:00 and 1.98 ± 2.37 ng/ml at 20:00 (average $\pm$ S.D.). The time course of T is also presented in Fig. 6. Four out of five rats had higher T concentrations in the afternoon and lower T concentrations in the evening. The time course was similar to those observed in 40-day-old Sprague–Dawley-descended rats [16] or 50-day-old Wistar rats [17]. The circadian rhythm of T in rats was confirmed and T concentration determination using the present method was proven to be applicable.

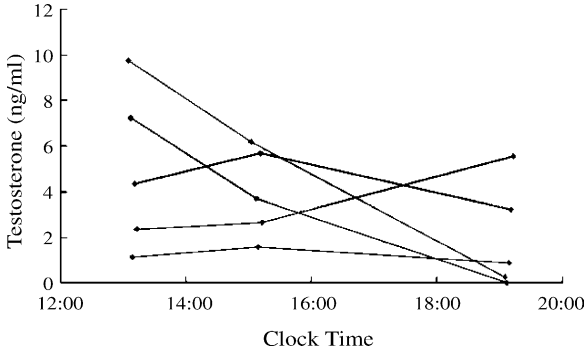

Fig. 6. Intra-day change in plasma testosterone levels in male 8-week-old Crl:CD(SD) rats. Blood samples (200 μl) were collected from the jugular veins of rats and 50 μl of plasma was subjected to analysis.

4. Conclusion

A sensitive assay for measuring T concentrations in rat plasma samples was developed and validated. With this assay, accurate T concentration determinations utilizing only 50 μl of rat blood plasma are now possible. The sensitivity proved to be adequate, covering the concentration range of 0.2–20 ng/ml, considering the endogenous nature of the analyte. The method is also considered to be robust because extraction recovery was complete and matrix effects were negligible. In addition, the use of ethoxime acetate derivatives would be useful in the sensitive measurement of steroid concentrations using LC–APCI/MS/MS.

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